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Mini-review

Molecular insight into specific 14-3-3 modulators: Inhibitors and stabilisers of protein–protein interactions of 14-3-3

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ABSTRACT

The 14-3-3 protein family is implicated in several diseases and biological processes. Several recent reviews have summarised knowledge on certain aspects of 14-3-3 proteins, ranging from a historic overview to the structure, function and regulation. This review focuses on the structures and molecular recognition of the modulators by the 14-3-3 proteins, and small modifications of certain modulators are proposed where cocrystal structures have been reported. Our analysis opens up possibilities for the optimisation of the reported compounds. It is very timely to analyse the current status of recently developed modulators given that the field has seen a lot of activity in recent years. This review provides an overview combined with a critical analysis of each class of modulators, keeping their suitability for future development in mind.

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1. Introduction

Protein–protein interactions (PPIs) play a significant role in many diseases. While a single polypeptide chain can have enzymatic or structural activity, interactions with other proteins allow an infinite variety of ways to modulate the activity. It is assumed that between 130,000 and 650,000 PPIs exist, making PPIs important therapeutic targets. This especially holds for diseases, which are difficult to treat, for example when targets like ion channels, enzymes and GPCRs have not yet been identified or are not present at all [1].

One class of proteins in which PPIs play an important role is the 14-3-3 protein family. This protein family consists of seven isoforms in humans: beta (β), epsilon (ε), eta (η), gamma (γ), tau (τ), sigma (σ) and zeta (ζ). Each of these human isoforms is encoded by a distinct genetic sequence. While plants have more than ten isoforms, eukaryotic micro-organisms have fewer isoforms, for instance, two isoforms have been reported in yeasts [2]. All isoforms have a high conformational and functional conservation. The two isoforms found in yeast can be interchanged with plant or mammalian isoforms and still be active [2]. It has been shown that

the binding groove of all isoforms has three conserved binding motifs: RSXpSXP (mode 1), RXXXpSXP (mode 2) and pS/TX-COOH (mode 3), in which pS stands for a phosphoserine residue. [3,4] 14-3-3 proteins primarily form dimers, which both feature a U-shaped binding groove [5,6]. A superimposition of the seven isoforms is shown in Fig. 1, illustrating the conserved conformation of the monomers [7]. Wang and Shakes have made multiple alignments of forty-six sequences of plant, animal and fungal species to determine the molecular evolution of the 14-3-3 protein family [8]. Wilkert *et al.* also show a sequence conformation among the 14-3-3 proteins [9].

The 14-3-3 monomers are acidic (pI ≈ 4.6) [10], have a molecular weight of 25–30 kDa and can combine to form homo- and heterodimers [11]. The non-trivial name 14-3-3 was assigned to this protein family by the researchers who first identified it from a classification study on brain proteins, due to position of the bands on 2D diethylaminoethyl (DEAE)-cellulose chromatography and starch gel electrophoresis.

The 14-3-3 family is implicated in several diseases and biological processes. Studies on binding partners of the 14-3-3 proteins have revealed over 500 proteins, and this number will continue to grow with ongoing research [12]. Among these binding partners are many biologically relevant targets. In some diseases, the 14-3-3 proteins have inhibitory effects, while in other cases they lead to stabilisation. It is even observed that binding of 14-3-3 enhances

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Fig. 1. Superimposition of monomers of the seven human isoforms.

the activity of the enzyme *N*-acetyltransferase (AANAT) [3]. Examples of such processes are: regulation of metabolism, signal transduction, cell-cycle control, apoptosis, protein trafficking, transcription, stress response and malignant transformation [5]. A few examples of the many diseases in which 14-3-3 proteins are involved are presented in Table 1.

In order to develop treatments for these diseases, it will be necessary to modulate PPIs of the relevant proteins. An approach to modulate the PPIs is by the use of small-molecule modulators. Modulators of the PPIs of 14-3-3 with partner proteins can come in two types: inhibitors or stabilisers. Small-molecules modulators are usually derived from natural products such as proteins, peptides or secondary metabolites. These modulators have complex structures, making them specific and selective for 14-3-3 proteins [20]. As the isoforms are encoded by distinct genes, they have a unique but similar amino acid sequence. Due to this uniqueness it might be possible to selectively modulate a single isoform, leaving the other isoforms untouched. Notably, this selectivity is essential from a therapeutic point of view, as the drug should not disturb any other biological processes. Research into the discovery and optimisation of small-molecule modulators should therefore take the selectivity of the compounds into consideration, wherever applicable.

Here, we systematically explore the binding of small-molecule modulators of 14-3-3 proteins at the molecular level, that is, the exact binding of the modulators to the amino acid residues lining the binding groove, aiming to provide an insight into the way these modulators work. For almost all modulators in this review of which a crystal structure is known, we propose modifications based on a structure-based analysis. Therefore, this review gathers knowledge, which can be used for structure-based design of (new) modulators. Several other recent reviews have summarised knowledge on other

aspects of 14-3-3 proteins: the structural basis [6], insights from genome-wide studies [21], a historic overview [22], the structure, function and regulation [7], an update on 14-3-3 sigma related to human cancer [11] and small-molecule modulators with a focus on the discovery and the biochemical relevance of the compounds [12].

2. Structure-based optimisation

There are cocrystal structures available of almost all 14-3-3 isoforms (some of which are in complex with interaction partners). 14-3-3 can therefore be conveniently targeted using a structure-based approach. The analysis of the binding modes of the modulators described in this review gives insight into where the compounds might be extended or modified in order to achieve higher affinity.

For the known cocrystallised modulators, we suggest small modifications, based on a first inspection of the experimentally determined binding mode analysed *in silico*. More advanced docking studies and biochemical evaluations should be performed to confirm the improved affinities. The computer programme SeeSAR was used for the evaluation, using the in-built scoring function HYDE [23–25]. As explained by Reulecke *et al.*, HYDE focusses on hydration and desolvation, taking into consideration the local hydrophobicity, solvent-accessible surface and contact surface area. It calculates this affinity for every atom present in the interface [25].

3. Inhibitors

The first compounds reported to inhibit the PPIs of 14-3-3 with partner proteins were **1** and its dimer difopein (dimeric 14-3-3 peptide inhibitor) (**2**) (Fig. 2) [26]. The former, a 20-amino acid peptide of Mw 2309.6, was identified by Wang *et al.* from a phage display to 14-3-3(τ) [27]. Petosa *et al.* showed *via* a crystal structure (Fig. 3) that R18 binds in the amphipathic groove and that the central WLDLE motif is hosted in the part of the pocket that binds phosphonic acids, also called the phospho-accepting pocket [28]. Moreover, the peptide does not require phosphorylation to bind to 14-3-3, as the negatively charged Asp and Glu mimic the contact points of phosphoserine [7,26]. As seen from HYDE scoring in the programme SeeSAR, there are multiple favourable and some unfavourable interactions between **1** and 14-3-3(ζ) (Fig. S1 in the supporting information) [23,24]. The C-terminal leucine of **1** is involved in lipophilic interactions with Gly169 and Ile217. The carboxylic acid terminal group of **1** shows electrostatic interactions with Arg56 and Arg127. Also the oxygen of the left-most amide bond shows to lose energy due to desolvation.

Compound **1** does not have selectivity between isoforms of 14-3-3. However, it is selective for 14-3-3 proteins [26]. Binding by **1** inhibits binding of partners like Raf-1, ASK1 and exoenzyme S from binding to 14-3-3 *in vitro*. Fu and Masters reported that **2**, enhances the ability of the antineoplastic drug cisplatin to kill cells. Compound **2** can bind competitively to one or two monomers of 14-3-3 given that the two **1** repeats are separated by a flexible linker. Cao

Table 1

Few examples of many diseases in which 14-3-3 proteins are involved.

14-3-3 isoform	Disease/function	Binding partner
(σ) and (ζ)	Noonan Syndrome [13]	C-RAF
(ζ)	Cell death [14,15]	Pathogenic protein Exoenzyme S
(σ) specifically	Oncogenesis and cellular response in DNA damage [9,16]	p53
all	Mutant (breast) cancer [17,18]	RAF
(σ) and (ζ)	Alzheimer's [19]	Tau

Pro-His-Cys-Val-Pro-Arg-Asp-Leu-Ser-Trp-Leu-Asp-Leu-Glu-Ala-Asn-Met-Cys-Leu-Pro

Fig. 2. Structure of the WLDLE motif, which is found in the phospho-accepting pocket of 14-3-3 proteins [27] and the sequence of **1**, highlighted is the WLDLE motif.

Fig. 3. Cocrystal structure of 14-3-3(ζ) with **1** (PDB code: 1A38). Colour code: protein surface: grey; interacting residues: sticks; inhibitor skeleton: C: yellow; N: blue; O: red, Cl: green; H-bonds, below 3.2 Å: green, dashed lines. This colour code is maintained throughout this review [28]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al. showed that **2**, as a general antagonist of 14-3-3, effectively hinders proliferation and triggers apoptosis of tumour cells, which were implanted in nude mice. *Via* flow cytometric analysis it was shown that apoptosis is time-dependent [29].

The first small-molecule inhibitor of 14-3-3 proteins was compound **3** (Fig. 4), which specifically and competitively inhibits 14-3-3/ligand binding *in vitro*. Wu *et al.* demonstrated that **3** also causes apoptosis and cell-cycle arrest in mammals. The central motif of **3** is a phosphorylated peptide-like small molecule; its non-phosphorylated analogue shows no activity in competing with 14-3-3(σ)/ligands. Molecular docking was used by the authors to confirm the binding mode of **3** with 14-3-3(σ); no cocrystal

structure is available [30]. Optimisation of **3** led to compound **4**. This prodrug is a phosphoserine mimetic and potent 14-3-3 inhibitor. When compound **4** is used as a prodrug, the active compound, the phosphonic acid is delivered more effectively into the cells [31].

Almost concurrently with the discovery of **4**, Corradi *et al.* disclosed the non-peptidic small-molecule **5**, an inhibitor of the 14-3-3/c-Abl complex (Fig. 5). The authors used an *in silico* virtual screening campaign and docking simulations, based on a structure-based pharmacophore model, against 200,000 compounds. Compound **5** can be used to treat Bcr-Abl-associated diseases, which are resistant to traditional drugs [32]. Unfortunately, it was found that **5** is chemically unstable, as it cyclises at room temperature [33]. The authors carried out a virtual-screening campaign with the aim of identifying compounds that score better than **5**. Compounds **6** and **7** emerged as hits that are cytotoxic against Bcr-Abl-expressing Ba/F3 cells, and according to molecular modelling bind to 14-3-3(σ) in a manner similar to that of **5** and **1** [33].

Another small-molecule inhibitor of all 14-3-3 isoforms in 14-3-3 PPIs, FOBISIN101 (**8**), was reported in 2011 (Fig. 6) [34]. FOBISIN (FOurteen-three-three Binding Small molecule INhibitor) 101 is a phosphoSer/Thr-mimetic agent, which upon X-ray irradiation covalently binds to 14-3-3(ζ) at Lys120. Furthermore, it has lipophilic interactions with Pro167, Gly171 and Ile219 and polar interactions to the flexible Lys49. Zhao *et al.* observed a colour change from bright orange before X-ray irradiation to brownish then yellowish and finally colourless after irradiation. In addition to this covalent linkage, which plays a role in its inhibitory activity, the authors suggest that the phosphate moiety might play a critical role. This conclusion was drawn from the observation that a non-phosphorylated derivative of **8** is significantly less effective in blocking 14-3-3/ligand binding. Mutation of Lys120 to Glu was shown to inactivate 14-3-3(ζ), this was observed in an assay to determine the activation of ExoS ADP-ribosyltransferase. Therefore, this indicates that Lys120 plays an important role in the binding to ligands/modulators [34]. The authors noted that another covalent linkage might also occur through imine formation, which was proposed later by Röglin *et al.* and is shown in Fig. 7. Röglin *et al.* propose a likely mechanism involving imine hydrolysis to a

Fig. 4. Inhibitors **3** and prodrug **4**, identified *via* a small-molecule microarray (SMM) technique consisting of fragment-based high-throughput identification combined with a reconstitution step and biological validation [30].

Fig. 5. Inhibitors 5, 6 and 7, identified via a virtual screening campaign [32].

Fig. 6. Inhibitors FOBISIN101 (8) and the covalent adduct 9 with 14-3-3(ζ). Compound 8 was identified from a fluorescence polarisation-based 14-3-3 binding assay, screening compounds that disrupt the 14-3-3(γ)-pS259-Raf-1 peptide complex [34].

Fig. 7. Cocrystal structure of 9: compound 8 covalently linked to Lys122 of 14-3-3(σ) via an imine bond (PDB code: 3U9X) [35].

pyridoxal-phosphate (PLP) intermediate followed by imine formation with Lys122 of 14-3-3(ζ) [35]. The differences in experimental conditions appear to determine whether the covalent linkage takes place. With the Cu-K α edge radiation they did not observe a colour change/covalent linkage as previously described. However, synchrotron irradiation did result in the covalent

modification at Lys120 as described before. They agree that the risk of nonspecific imine formation might be prevented by eliminating the aldehyde functionality, thereby making it a more potent class of compounds [36].

Shortly after the publication on 8, Park *et al.* reported on xanthine (10) and lacosamide (11) derivatives as specific binders of 14-3-3(ζ) (Fig. 8). Using *in vitro* labelling, they showed that the enantiomer *R*-11 binds specifically to 14-3-3(ζ). The stereoisomer *S*-11 was found to be less active, and *R*-12 outcompetes *R*-11 for binding to 14-3-3(ζ). The authors were unable to explain the isoform selectivity, cocrystallographic data could help to gain a better understanding [37].

A high-throughput screen using an *in vitro* fluorescence-polarisation assay afforded another class of scaffolds that modulate 14-3-3/ligand interactions, namely compounds with the core structure 13 (Fig. 9) [38]. The main part of the PRLX-scaffold is a 1,2,4-thiadiazole, decorated with substituents (R), which were not specified as they were patented compounds. There is no X-ray crystal structure published with these modulators. The authors used multiple biophysical assays to assess the activity of the compounds, starting with isolated cells and followed by intact tissue. Five different 14-3-3 isoforms from *E. coli* ((β , ζ , η , ϵ and γ) were tested and it was found that the compounds had highest binding affinity for the (γ) isoform [38]. The origin of this specificity was not investigated in detail. The thiadiazole scaffold of compound 13 (Fig. 4) inhibits the interaction of 14-3-3(γ) and a phosphorylated HSP20 peptide (6-FAM-WLRRAPSA).

In 2011, the inhibitor 14 was identified in a study aimed at making derivatives of the natural product class of the moverastins,

Fig. 8. Modulator Xanthine (**10**) and inhibitors lacosamide (R)- and (S)-**11** and derivative (R)-**12**. 14-3-3 was identified as a binding partner to these compounds from a ligand affinity and chemical reporter strategy [37].

Fig. 9. Inhibitor scaffold **13** identified by high-throughput screening [38].

Fig. 10. Inhibitor **14**, a moverastin derivative that targets 14-3-3(ζ) [39].

which target cell migration of tumour cells for a possible treatment of tumour metastasis. Compound **14** (Fig. 10) was found to interact differently than moverastins, therefore suggesting a different target than the original target of the study, farnesyltransferase (FTase) [39]. A follow-up study confirmed 14-3-3(ζ) as the target, and from the seven human isoforms it was found that (ζ) had the strongest binding affinity. The authors suggest that this selectivity stems from the fact that the C-terminal region of 14-3-3 is involved in the binding, which is the most variable region of the isoforms. Compound **14** presumably inhibits cell migration by competing with the 14-3-3(ζ)/Tiam1 complex [40].

In 2012, Yan *et al.* identified blapsins A (**15**) and B (**16**) as potent small-molecule inhibitors. These compounds (Fig. 11) were isolated from *Blaps japonesis*, characterised and evaluated for their inhibitory potencies on 14-3-3(γ) [41]. They were found to inhibit 14-3-3 binding to the fluorescence-labelled TMR-Raf pSer259 peptide, used for monitoring protein interactions [42].

A virtual-screening campaign and subsequent experimental

validation revealed compound **17** (Fig. 12) as a potent inhibitor of 14-3-3(ζ) [43]. Compound **17** has a structure similar to that of **15**, except that **17** has a phosphonic acid, which serves as an anchor in the binding pocket of 14-3-3. The authors screened the ZINC *all now* subset in order to find a non-peptidic and non-covalently binding small-molecule inhibitor of the extracellular PPI of 14-3-3 with the membrane receptor aminopeptidase N (APN). In addition to disruption of the 14-3-3(σ)/APN complex, compound **17** was also found to inhibit the overexpression of matrix metalloproteinase (MMP)-1 in human fibroblasts, which was induced by signalling of 14-3-3 proteins. The binding mode of **17** to 14-3-3 proteins was validated by solving a cocrystal structure in complex with 14-3-3(σ) (Fig. 13) [43].

Compound **17** binds strongly due to the phosphonic acid. Analysis with the programme SeeSAR revealed that both chloride atoms are involved in lipophilic interactions with Arg60, Arg56 and Ala57, the oxygen atoms at the phosphonic acid anchor are involved in strong H-bonding interactions with Arg129 and Arg56. The carbon atom *para* to the phosphonic acid does not contribute to affinity. This problem might be overcome by installing a hydroxyl moiety on that carbon, as was modelled in the SeeSAR programme (see the supporting information Figs S2 and S3).

In 2014, Glas and coworkers reported that constrained peptides can serve as inhibitors of pathogenic PPIs. They used target-adapted cross-links to stabilise secondary structures in order to inhibit the interaction of 14-3-3 with virulence vector exoenzyme S (ExoS) [15]. The ES_p sequence was used as a starting point for the design of macrocyclic structures. This ES_p sequence, ⁴²⁰QGLLDALDLAS⁴³⁰, had previously been identified as a binding motif [14]. The authors compared the binding mode of their cross-linked peptide, **18** (Fig. 14), to that of ES_p by solving the crystal structure of both ligands with 14-3-3(ζ) (Fig. 15 and supporting information Fig. S4). Both ligands were shown to lie in the same binding site, **18**, however, has a 20-fold higher binding affinity. The authors suggest, *via* NMR, CD and ITC experiments, that the improved binding entropy for the cross-linked peptide is due to the reduction of conformational flexibility [15].

A novel modulator of 14-3-3/ligand complexes was introduced by Milroy *et al.* They started with the stabiliser Fusicoccin A (FC)

Fig. 11. Inhibitors Blapsin A (**15**) and Blapsin B (**16**), isolated from *Blaps japonesis* [41].

Fig. 12. Structure of virtual-screening hit **17**, a potent 14-3-3 inhibitor [43].

Fig. 13. Crystal structure of virtual-screening hit **17** in complex with 14-3-3(σ) (PDB code: 4DHU) [43].

Fig. 14. Structure of the constrained peptide **18**, an inhibitor of 14-3-3(ζ) [15].

(**19**) (Fig. 17) in order to design inhibitors, since they found that the region on 14-3-3 where **19** binds partially overlaps with the region where known inhibitors bind. The part of the active site from which stabilisation is observed lies next to the part where inhibition is observed, that is, there is a small interface between the modulating effects of these parts. The Tau pSer214 epitope was modified with different amines to obtain higher affinity. Milroy *et al.* hypothesised that using the OMe substituent of **19** would give high affinities due to interactions with the FC pocket, consisting of Phe, Met and Lys residues [19].

The Tau epitope, AcNH-RTPpSLPTP-OH, decorated with compound **20** (Fig. 16) showed to have good affinities for 14-3-3 due to the OMe group. Yet, tau epitope expanded with **21** emerged as the most active inhibitor, the bulky aromatic groups increased the affinity. This compound inhibits the binding of phosphorylated full-length Tau (protein) to 14-3-3. It has a 225-fold higher affinity

than the Tau epitope. Inhibitors of 14-3-3/Tau might be useful in the treatment of Alzheimer's disease. However, the cocrystal structure they reported is in complex with amine **20**, which has a fifteen-fold worse IC₅₀ value than **21** (see the supporting information Fig. S5) [19].

4. Stabilisers

The natural product Fusicoccin A (**19**) was reported as the first stabiliser of PPIs of 14-3-3/ligands. This diterpene glycoside, member of a class of diterpenes bearing a 5-8-5 ring structure called fusicocanes, was found to bind to 14-3-3 receptors [44]. **19** molecularly glues together the 14-3-3/plasma membrane ATPase complex (PMA), while it does not bind to the individual partners [45]. The PMA is bound at the C-terminus via a phosphorylated Thr. The scaffold of **19** provides a starting point for the design of further stabilisers of 14-3-3/ligand complexes, as it interacts on multiple sites with both protein and ligands.

19 was found to not only stabilise the plant plasma membrane pump but also the binding of 14-3-3 to estrogen receptor alpha (ER α). The ER α is an anticancer target given that stabilising the 14-3-3/ER α complex has a downstream effect and leads to decreased cell proliferation [18].

Binding of **19** to PMA2 can probably be enhanced by changing the oxygen on the oxane ring for a carbon atom (Fig. 17 and supporting information Figs. S6–S8). In this case, the programme SeeSAR predicts a better affinity, and Hyde evaluation only reveals

favourable interactions. A similar improvement can be achieved in **19** with the 14-3-3/cystic fibrosis transmembrane conductance regulator (CFTR) complex: the oxygen atom bridging the two rings is found to partially disrupt binding. The binding is predicted to improve when the oxygen is replaced by a carbon atom (Fig. 18 and supporting information Figs. S9–S11).

Using **19** as a starting point, Anders *et al.* made a semisynthetic derivative (**22**) (Fig. 19). This derivative targets 14-3-3 in the human potassium channel TASK-3 and interacts in the mode 3 motif of the conserved binding groove. **22** leads to a 19-fold increased binding of 14-3-3 to TASK-3 [48].

This potent stabiliser has some clashes of the oxygen atom of the oxane ring with the carbonyl of Asn42, as observed from analysing the cocrystal structure. This unfavourable interaction can be avoided by replacing the oxygen for a carbon atom, which according to the Hyde-scoring of SeeSAR, would give better interactions and a

Fig. 15. Cocystal structure of cross-linked peptide **18** with 14-3-3(ζ) (PDB code: 4N84) [15].

better affinity than **22** (see supporting information Figs. S12–S14).

Cotylenin A (CN-A) (**23**) is a natural product closely related to **19**. Compound **23** has anti-tumour activity by stabilising certain PPIs involved in human cancers. It is assumed that the hydroxyl moiety on the cyclopentenyl ring in **19** might determine the structural specificity compared to **23** and related fusicocanines [49]. Four years after the work of Ottmann, Molzan *et al.* reported that the 14-3-3/C-RAF complex is stabilised by **23** (Fig. 20). Cotylenin A interacts with pSer233 and pSer259 of C-RAF. At these positions, 14-3-3 binding inhibits C-RAF, whereas at pSer621, 14-3-3 binding activates C-RAF [50,51]. The *in vivo* activity of **23** on 14-3-3/C-RAF is increased compared to the *in vitro* activity. This difference indicates that other

processes are also influenced by **23** [17].

Compound **23** displays an unfavourable interaction at the oxygen atom bridging the two ring structures. Substituting this oxygen for a nitrogen atom could increase the affinity by having an additional H-bond towards Asp-213 (Figs. S15–S17 in the supporting information). The two six-membered rings are now less involved in binding, however changing the bridging oxygen atom for a nitrogen atom should lead to an increased affinity.

In 2010, Rose *et al.* identified small-molecule stabilisers of 14-3-3 PPIs, namely a tri-substituted pyrrolinone (**24**), and a dipeptide epibestatin (**25**). The authors screened a 37,000-membered compound library using green fluorescent protein (GFP) fused with 14-3-3 against binding to surface-immobilised glutathione *S*-transferase fused with PMA2-CT52. The selective stabilisation of 14-3-3/PMA2 by **25** displays a slower association than **19** and **24**, but a similar K_d to **19**. In other words, stabilising the PPI with **25** is relatively slow but once it has been established it will bind strongly (Fig. 21) [52]. It was found that compound **26** has a stabilising effect on 14-3-3/PMA2 that is three times stronger than that of **24** (Figs. 22 and 23), presumably because more contact points with the PMA2 residues are established [53].

The programme SeeSAR was used to suggest some modifications [23]. Substituting the free amine in epibestatin (**25**) by a carbon atom, for instance, enhances the binding to the PMA moiety. However, this still gives clashes; changing it into a hydroxyl group makes it a bit smaller and again an H-bond acceptor/donor. With an alcohol moiety the affinity most probably increases again, but clashes are still seen with Leu929 and His930 (see Figs. S18–S20 in the supporting information).

The hydroxyl group of the central five-membered ring in **24** appears to have some unfavourable interactions with Val45 and Asn49. Replacing the hydroxyl group for an amine improves the affinity a bit, going to fluoride improves the binding even more (Figs. S21–S23 in the supporting information).

Mizoribine (**27**), or bredinin (Fig. 24), is a compound isolated from *Eupenicillium brefeldianum* and found to have activities in

Fig. 16. Cocystal structure of 14-3-3(σ) and phosphorylated Tau epitope with amine **20**, identified using a structure-based drug design (SBDD) approach (PDB code: 4Y3B) [19].

Fig. 17. Crystal structure of the stabiliser Fusicoccin (**19**)-plasma membrane ATPase complex 2-C52 in the tobacco 14-3-3c isoform (PDB code: 2098) [46].

Fig. 18. Fusicoccin A (**19**) and cystic fibrosis transmembrane conductance regulator R6-domain with 14-3-3(ζ) (PDB code: 5D3F) [47].

multiple animal studies as well as an immunosuppressive activity [54]. This imidazole nucleoside enhances the interaction of glucocorticoid receptor (GR) with 14-3-3(η) *in vitro* [55]. Mizoribine has been an approved drug in Japan for many years for the treatment of lupus nephritis, rheumatoid arthritis and for immunosuppressive therapy after renal transplantation [55].

The most recently published small-molecule stabiliser **28** holds promise to be used in new therapeutic approaches to target Gab2-related cancers. Gab2 is a signalling protein [56], which is correlated to the mitogen-activated protein kinase (MAPK) pathways involved in certain cancer types (e.g., breast cancer) [57]. Bier *et al.* showed in an elaborate study that the FC-derivative **28** stabilises the binding of pT391 peptides, which are derived from the binding moiety of Gab2 (Fig. 25). The stabilisation is specific for the pT391 motif, as no enhanced binding was observed for the other phosphorylation site of Gab2, the Gab2pS210 binding motif. The authors examined the binding interactions of Gab2pT391 and Gab2pS210 via cocrystal structures [58]. It was shown that, in the pT391 motif,

Fig. 19. Cocrystal structure of Fusicoccin-derivative (**22**) bound to 14-3-3(σ) (PDB code: 3SPR) [48].

Fig. 20. Cocrystal structure of stabiliser Cotylenin A (23) and C-RAF in 14-3-3(ζ) (PDB code: 4IHL) [17].

Fig. 21. Crystal structure of stabiliser epibestatin (25)-plasma membrane ATPase complex 2-CT30 in 14-3-3 (PDB code: 3M50). 25 was identified by screening a compound library against binding of 14-3-3 to plasma membrane ATPase complex 2-C52 [52].

Fig. 22. Stabiliser 24 and 26, identified by screening a compound library against binding of 14-3-3 to plasma membrane ATPase complex 2-CT52 [52].

Leu392 of Gab2 can be best accommodated in the hydrophobic contact surface of Leu172, Ile217 and Leu220 of 14-3-3(ζ). Gab2 motifs are anchored through the phosphonic acid in the binding triad of Arg56, Arg127 and Tyr128, as is frequently observed and described in this review. The cocrystal structure of 14-3-3(ζ) with Gab2 and 28 is given in Fig. 26. The hydrophobic pocket in 14-3-3(ζ), consisting of the following amino acids Val46, Phe117, Met121, Pro165, Ile166, Gly169 and Ile217, is accommodated by the terpenoid core of 28 and by Leu392 of Gab2. The Gab2pT391 peptide has a sharp turn, most probably induced by Pro393, which therefore gives space for 28 to bind and stabilise the complex. This turn is not observed in Gab2pS210, which prompts the authors to postulate that the difference of one or two amino acids in the 14-3-3 recognition motifs of Gab2 could be used to design PPI-target-specific stabilisers [58].

The program SeeSar was used to analyse the binding affinity of 28 to Gab2pT391 peptide and 14-3-3(ζ). The methoxy carbon atom at the terpenoid core has strong interactions with Met121 and

Fig. 23. Cocystal structure of stabiliser **24** and plasma membrane ATPase complex 2-CT30 in 14-3-3 (PDB code: 3M51) [52].

Fig. 24. Stabiliser mizoribine (**27**), isolated from *Eupenicillium brefeldianum*, stabilises the glucocorticoid receptor (GR) with 14-3-3(η) [54].

Phe117. The remaining carbon atoms are also involved in hydrophobic interactions with the protein. For example, the isopropyl moiety has strong interactions with Asp213, Ile217 and Leu392. However, the bridging oxygen atom towards the two six-membered rings shows to be non-favourable. It forms an intramolecular H-bond, but it loses affinity due to desolvation. Substituting this oxygen atom for a carbon atom might improve the

binding affinity, as this carbon atom could then have lipophilic interactions with Asp213. The terpenoid core should remain in the hydrophobic part, and the two six-membered rings, which are solvent exposed move slightly. (Figs. S24–S26 in the supporting information) [59].

5. Conclusions

Research has focussed, for almost 20 years now, on the development of 14-3-3 inhibitors and stabilisers. We analysed the structures of the reported inhibitors and stabilisers. Visual inspection of the cocystal structures of the published small-molecule modulators at the molecular level aided by the programme SeeSAR has led to the proposal of small modifications, which might improve the binding affinity. The knowledge gained from exploring the cocystal structures sets the stage for structure-based design of (new) modulators. Designing molecules to stabilise or even activate 14-3-3/partner protein complexes has the potential to become an important part of modulating 14-3-3 PPIs, since research has focussed so far more on inhibiting PPIs.

Fig. 26. Cocystal structure of stabiliser **28** and Gab2pT391 peptide in 14-3-3(ζ) (PDB code: 5EXA) [58].

Fig. 25. Stabiliser **28**, which stabilises the complex of Gab2pT391 peptide and 14-3-3(ζ) [58].

Since there are seven isoforms, with a high degree of conservation, it might be wiser to target the interaction partner rather than 14-3-3. This approach might lead to more specific small-molecule modulators that target a specific PPI instead of all PPIs from the 14-3-3 protein. This would require a paradigm shift, as research has focussed on binding to 14-3-3. Focussing on the interaction partner might give more freedom and creativity when aiming to influence PPIs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2017.04.058>.

References

- [1] M. Skwarczynska, C. Ottmann, Protein–protein interactions as drug targets, *Future Med. Chem.* 7 (2015) 2195–2219, <http://dx.doi.org/10.4155/fmc.15.138>.
- [2] G. Tzivion, J. Avruch, 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation, *J. Biol. Chem.* 277 (2002) 3061–3064, <http://dx.doi.org/10.1074/jbc.R100059200>.
- [3] S. Ganguly, J.L. Weller, A. Ho, P. Chemineau, B. Malpau, D.C. Klein, Melatonin synthesis: 14-3-3-dependent activation and inhibition of arylalkylamine N-acetyltransferase mediated by phosphoserine-205, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 1222–1227, <http://dx.doi.org/10.1073/pnas.0406871102>.
- [4] B. Coblitz, M. Wu, S. Shikano, M. Li, C-terminal binding: an expanded repertoire and function of 14-3-3 proteins, *FEBS Lett.* 580 (2006) 1531–1535, <http://dx.doi.org/10.1016/j.febslet.2006.02.014>.
- [5] X. Yang, W.H. Lee, F. Sobott, E. Papagrigoriou, C.V. Robinson, J.G. Grossmann, M. Sundström, D.A. Doyle, J.M. Elkins, Structural basis for protein–protein interactions in the 14-3-3 protein family, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 17237–17242, <http://dx.doi.org/10.1073/pnas.0605779103>.
- [6] T. Obsil, V. Obsilova, Structural basis of 14-3-3 protein functions, *Semin. Cell Dev. Biol.* 22 (2011) 663–672, <http://dx.doi.org/10.1016/j.semcdb.2011.09.001>.
- [7] H. Fu, R.R. Subramanian, S.C. Masters, 14-3-3 proteins: structure, function, and regulation, *Annu. Rev. Pharmacol. Toxicol.* 40 (2000) 617–647, <http://dx.doi.org/10.1146/annurev.pharmtox.40.1.617>.
- [8] W. Wang, D.C. Shakes, Molecular evolution of the 14-3-3 protein family, *J. Mol. Evol.* 43 (1996) 384–398.
- [9] E.W. Wilkert, R.A. Grant, S.C. Artim, M.B. Yaffe, A structural basis for 14-3-3sigma functional specificity, *J. Biol. Chem.* 280 (2005) 18891–18898, <http://dx.doi.org/10.1074/jbc.M500982200>.
- [10] M.B. Yaffe, K. Rittinger, S. Volinia, P.R. Caron, A. Aitken, H. Leffers, S.J. Gamblin, S.J. Smerdon, L.C. Cantley, The structural basis for 14-3-3:phosphopeptide binding specificity, *Cell* 91 (1997) 961–971, [http://dx.doi.org/10.1016/S0092-8674\(00\)80487-0](http://dx.doi.org/10.1016/S0092-8674(00)80487-0).
- [11] P. Mhawech, 14-3-3 proteins—an update, *Cell Res.* 15 (2005) 228–236, <http://dx.doi.org/10.1038/sj.cr.7290291>.
- [12] C. Ottmann, Small-molecule modulators of 14-3-3 protein–protein interactions, *Bioorg. Med. Chem.* 21 (2013) 4058–4062, <http://dx.doi.org/10.1016/j.bmc.2012.11.028>.
- [13] M. Molzan, B. Schumacher, C. Ottmann, A. Baljuls, L. Polzien, M. Weyand, P. Thiel, R. Rose, M. Rose, P. Kuhenne, M. Kaiser, U.R. Rapp, J. Kuhlmann, C. Ottmann, Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling, *Mol. Cell. Biol.* 30 (2010) 4698–4711, <http://dx.doi.org/10.1128/MCB.01636-09>.
- [14] C. Ottmann, L. Yasmin, M. Weyand, J.L. Veessenmeyer, M.H. Diaz, R.H. Palmer, M.S. Francis, A.R. Hauser, A. Wittinghofer, B. Hallberg, Phosphorylation-independent interaction between 14-3-3 and exoenzyme S: from structure to pathogenesis, *EMBO J.* 26 (2007) 902–913, <http://dx.doi.org/10.1038/sj.emboj.7601530>.
- [15] A. Glas, D. Bier, G. Hahne, C. Rademacher, C. Ottmann, T.N. Grossmann, Constrained peptides with target-adapted cross-links as inhibitors of a pathogenic protein–protein interaction, *Angew. Chem. - Int. Ed.* 53 (2014) 2489–2493, <http://dx.doi.org/10.1002/anie.201310082>.
- [16] H. Hermeking, C. Lengauer, K. Polyak, T.-C. He, L. Zhang, S. Thiagalingam, K.W. Kinzler, B. Vogelstein, 14-3-3σ is a p53-regulated inhibitor of G2/M progression, *Mol. Cell.* 1 (1997) 3–11, [http://dx.doi.org/10.1016/S1097-2765\(00\)80002-7](http://dx.doi.org/10.1016/S1097-2765(00)80002-7).
- [17] M. Molzan, S. Kasper, L. Röglin, M. Skwarczynska, T. Sassa, T. Inoue, F. Breitenbuecher, J. Ohkanda, N. Kato, M. Schuler, C. Ottmann, Stabilization of physical RAF/14-3-3 interaction by Cotylenin A as treatment strategy for RAS mutant cancers, *ACS Chem. Biol.* 8 (2013) 1869–1875, <http://dx.doi.org/10.1021/cb4003464>.
- [18] I.J. De Vries-van Leeuwen, D. da Costa Pereira, K.D. Flach, S.R. Piersma, C. Haase, D. Bier, Z. Yalcin, R. Michalides, K.A. Feenstra, C.R. Jiménez, T.F. de Greef, L. Brunsvel, C. Ottmann, W. Zwart, A.H. de Boer, Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 8894–8899, <http://dx.doi.org/10.1073/pnas.1220809110>.
- [19] L.G. Milroy, M. Bartel, M.A. Henen, S. Leysen, J.M.C. Adriaans, L. Brunsvel, I. Landrieu, C. Ottmann, Stabiliser-guided inhibition of protein–protein interactions, *Angew. Chem. - Int. Ed.* 54 (2015) 15720–15724, <http://dx.doi.org/10.1002/anie.201507976>.
- [20] L.G. Milroy, T.N. Grossmann, S. Hennig, L. Brunsvel, C. Ottmann, Modulators of protein–protein interactions, *Chem. Rev.* 114 (2014) 4695–4748, <http://dx.doi.org/10.1021/cr400698c>.
- [21] G.P.H. van Heusden, 14-3-3 proteins: insights from genome-wide studies in yeast, *Genomics* 94 (2009) 287–293, <http://dx.doi.org/10.1016/j.ygeno.2009.07.004>.
- [22] A. Aitken, 14-3-3 proteins: a historic overview, *Semin. Cancer Biol.* 16 (2006) 162–172, <http://dx.doi.org/10.1016/j.semcancer.2006.03.005>.
- [23] BioSolveIT GmbH, SeeSAR 4.1.
- [24] BioSolveIT GmbH, HYDE binding assessment, <https://www.biosolveit.de/HYDE/>.
- [25] I. Reulecke, G. Lange, J. Albrecht, R. Klein, M. Rarey, Towards an integrated description of hydrogen bonding and dehydration: decreasing false positives in virtual screening with the HYDE scoring function, *Chem. Med. Chem.* 3 (2008) 885–897, <http://dx.doi.org/10.1002/cmdc.200700319>.
- [26] S.C. Masters, H. Fu, 14-3-3 proteins mediate an essential anti-apoptotic signal, *J. Biol. Chem.* 276 (2001) 45193–45200, <http://dx.doi.org/10.1074/jbc.M105971200>.
- [27] B. Wang, H. Yang, Y.S. Liu, T. Jelinek, L. Zhang, E. Ruoslahti, Isolation of high-affinity peptide antagonist of 14-3-3 proteins by phage display, *Biochemistry* (1999) 12499–12504, 12499–12504.
- [28] C. Petosa, S.C. Masters, L.A. Bankston, J. Pohl, B. Wang, H. Fu, R.C. Liddington, 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphiphatic groove, *J. Biol. Chem.* 273 (1998) 16305–16310, <http://dx.doi.org/10.1074/jbc.273.26.16305>.
- [29] W. Cao, X. Yang, J. Zhou, Z. Teng, L. Cao, X. Zhang, Z. Fei, Targeting 14-3-3 protein, difopein induces apoptosis of human glioma cells and suppresses tumor growth in mice, *Apoptosis* 15 (2010) 230–241, <http://dx.doi.org/10.1007/s10495-009-0437-4>.
- [30] H. Wu, J. Ge, S.Q. Yao, Microarray-assisted high-throughput identification of a cell-permeable small-molecule binder of 14-3-3 proteins, *Angew. Chem.* 122 (2010) 6678–6682, <http://dx.doi.org/10.1002/ange.201003257>.
- [31] A. Arrendale, K. Kim, J.Y. Choi, W. Li, R.L. Geahlen, R.F. Borch, Synthesis of a phosphoserine mimetic prodrug with potent 14-3-3 protein inhibitory activity, *Chem. Biol.* 19 (2012) 764–771, <http://dx.doi.org/10.1016/j.chembiol.2012.05.011>.
- [32] V. Corradi, M. Mancini, F. Manetti, S. Petta, M.A. Santucci, M. Botta, Identification of the first non-peptidic small molecule inhibitor of the c-Abl/14-3-3 protein–protein interactions able to drive sensitive and Imatinib-resistant leukemia cells to apoptosis, *Bioorg. Med. Chem. Lett.* 20 (2010) 6133–6137, <http://dx.doi.org/10.1016/j.bmcl.2010.08.019>.
- [33] V. Corradi, M. Mancini, M.A. Santucci, T. Carlomagno, D. Sanfelice, M. Mori, G. Vignaroli, F. Falchi, F. Manetti, M. Radi, M. Botta, Computational techniques are valuable tools for the discovery of protein–protein interaction inhibitors: the 14-3-3σ case, *Bioorg. Med. Chem. Lett.* 21 (2011) 6867–6871, <http://dx.doi.org/10.1016/j.bmcl.2011.09.011>.
- [34] J. Zhao, Y. Du, J.R. Horton, A. K. Upadhyay, B. Lou, Y. Bai, X. Zhang, L. Du, M. Li, B. Wang, L. Zhang, J.T. Barbieri, F.R. Khuri, X. Cheng, H. Fu, Discovery and structural characterization of a small molecule 14-3-3 protein–protein interaction inhibitor, *Proc. Natl. Acad. Sci.* 108 (2011) 16212–16216, <http://dx.doi.org/10.1073/pnas.1100012108>.
- [35] L. Roglin, P. Thiel, O. Kohlbacher, C. Ottmann, Covalent attachment of pyridoxal-phosphate derivatives to 14-3-3 proteins, *Proc. Natl. Acad. Sci.* 109 (2012) E1051–E1053, <http://dx.doi.org/10.1073/pnas.1116592109>.
- [36] A.K. Upadhyay, J.R. Horton, Y. Du, Y. Bai, X. Cheng, H. Fu, Reply to Roglin et al.: synchrotron radiation-induced covalent modification of 14-3-3 by diazene compounds containing pyridoxal phosphate, *Proc. Natl. Acad. Sci.* 109 (2012), <http://dx.doi.org/10.1073/pnas.1203341109>. E1054–E1054.
- [37] K.D. Park, D. Kim, O. Reamtong, C. Evers, S.J. Gaskell, R. Liu, H. Kohn, Identification of a lacosamide binding protein using an affinity bait and chemical reporter strategy: 14-3-3σ, *J. Am. Chem. Soc.* 133 (2011) 11320–11330, <http://dx.doi.org/10.1021/ja2034156>.
- [38] S.S. An, P.S. Askovich, T.I. Zarebinski, K. Ahn, J.M. Peltier, M. von Rechenberg, S. Sahasrabudhe, J.J. Fredberg, A novel small molecule target in human airway smooth muscle for potential treatment of obstructive lung diseases: a staged high-throughput biophysical screening, *Respir. Res.* 12 (2011) 8, <http://dx.doi.org/10.1186/1465-9921-12-8>.
- [39] M. Sawada, S.I. Kubo, K. Matsumura, Y. Takemoto, H. Kobayashi, E. Tashiro, T. Kitahara, H. Watanabe, M. Imoto, Synthesis and anti-migrative evaluation of moverastin derivatives, *Bioorg. Med. Chem. Lett.* 21 (2011) 1385–1389,

- <http://dx.doi.org/10.1016/j.bmcl.2011.01.028>.
- [40] H. Kobayashi, Y. Ogura, M. Sawada, R. Nakayama, K. Takano, Y. Minato, Y. Takemoto, E. Tashiro, H. Watanabe, M. Imoto, Involvement of 14-3-3 proteins in the second epidermal growth factor-induced wave of Rac1 activation in the process of cell migration, *J. Biol. Chem.* 286 (2011) 39259–39268, <http://dx.doi.org/10.1074/jbc.M111.255489>.
- [41] Y.M. Yan, H.Q. Dai, Y. Du, B. Schneider, H. Guo, D.P. Li, L.X. Zhang, H. Fu, X.P. Dong, Y.X. Cheng, Identification of blapsins A and B as potent small-molecule 14-3-3 inhibitors from the insect *Blaps japonensis*, *Bioorg. Med. Chem. Lett.* 22 (2012) 4179–4181, <http://dx.doi.org/10.1016/j.bmcl.2012.04.027>.
- [42] Y. Du, S.C. Masters, F.R. Khuri, H. Fu, Monitoring 14-3-3 protein interactions with a homogeneous fluorescence polarization assay, *J. Biomol. Screen* 11 (2006) 269–276, <http://dx.doi.org/10.1177/1087057105284862>.
- [43] P. Thiel, L. Röglin, N. Meissner, S. Hennig, O. Kohlbacher, C. Ottmann, Virtual screening and experimental validation reveal novel small-molecule inhibitors of 14-3-3 protein-protein interactions, *Chem. Commun. (Camb)* 49 (2013) 8468–8470, <http://dx.doi.org/10.1039/c3cc44612c>.
- [44] C. Oecking, C. Eckerskorn, E.W. Weiler, The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins, *FEBS Lett.* 352 (1994) 163–166, [http://dx.doi.org/10.1016/0014-5793\(94\)00949-X](http://dx.doi.org/10.1016/0014-5793(94)00949-X).
- [45] C. Ottmann, S. Marco, N. Jaspert, C. Marcon, N. Schauer, M. Weyand, C. Vandermeeren, G. Duby, M. Boutry, A. Wittinghofer, J.L. Rigaud, C. Oecking, Structure of a 14-3-3 coordinated hexamer of the plant plasma membrane H(+)-ATPase by combining X-ray crystallography and electron cryomicroscopy, *Mol. Cell.* 25 (2007) 427–440, <http://dx.doi.org/10.1016/j.molcel.2006.12.017>.
- [46] B. Kostelecny, A.T. Saurin, A. Purkiss, P.J. Parker, N.Q. McDonald, Recognition of an intra-chain tandem 14-3-3 binding site within PKCepsilon, *EMBO Rep.* 10 (2009) 983–989, <http://dx.doi.org/10.1038/embor.2009.150>.
- [47] L.M. Stevers, C.V. Lam, S.F.R. Leysen, F.A. Meijer, D.S. van Scheppingen, R.M.J.M. de Vries, G.W. Carlile, L.G. Milroy, D.Y. Thomas, L. Brunsvel, C. Ottmann, Characterization and small-molecule stabilization of the multisite tandem binding between 14-3-3 and the R domain of CFTR, *Proc. Natl. Acad. Sci.* (2016), <http://dx.doi.org/10.1073/pnas.1516631113>. E1152–E1161.
- [48] C. Anders, Y. Higuchi, K. Koschinsky, M. Bartel, B. Schumacher, P. Thiel, H. Nitta, R. Preisig-Müller, G. Schlichthörl, V. Renigunta, J. Ohkanda, J. Daut, N. Kato, C. Ottmann, A semisynthetic fusicoccane stabilizes a protein-protein interaction and enhances the expression of K⁺ channels at the cell surface, *Chem. Biol.* 20 (2013) 583–593, <http://dx.doi.org/10.1016/j.chembiol.2013.03.015>.
- [49] C. Ottmann, M. Weyand, T. Sassa, T. Inoue, N. Kato, A. Wittinghofer, C. Oecking, A structural rationale for selective stabilization of anti-tumor interactions of 14-3-3 proteins by Cotylenin A, *J. Mol. Biol.* 386 (2009) 913–919, <http://dx.doi.org/10.1016/j.jmb.2009.01.005>.
- [50] N. Dumaz, R. Marais, Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras, *J. Biol. Chem.* 278 (2003) 29819–29823, <http://dx.doi.org/10.1074/jbc.C300182200>.
- [51] G. Tzivion, Z. Luo, J. Avruch, A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity, *Nature* 394 (1998) 88–92, <http://dx.doi.org/10.1038/27938>.
- [52] R. Rose, S. Erdmann, S. Bovens, A. Wolf, M. Rose, S. Hennig, H. Waldmann, C. Ottmann, Identification and structure of small-molecule stabilisers of 14-3-3 protein-protein interactions, *Angew. Chem. - Int. Ed.* 49 (2010) 4129–4132, <http://dx.doi.org/10.1002/anie.200907203>.
- [53] A. Richter, R. Rose, C. Hedberg, H. Waldmann, C. Ottmann, An optimised small-molecule stabiliser of the 14-3-3-PMA2 protein-protein interaction, *Chem. - A Eur. J.* 18 (2012) 6520–6527, <http://dx.doi.org/10.1002/chem.201103761>.
- [54] K. Kamata, M. Okubo, E. Ishigamori, Y. Masaki, H. Uchida, K. Watanabe, N. Kashiwagi, Immunosuppressive effect of bredinin on cell-mediated and humoral immune reactions in experimental animals, *Transplantation* 35 (1983) 144–149.
- [55] S. Takahashi, H. Wakui, J. a Gustafsson, J. Zilliacus, H. Itoh, Functional interaction of the immunosuppressant mizoribine with the 14-3-3 protein, *Biochem. Biophys. Res. Commun.* 274 (2000) 87–92, <http://dx.doi.org/10.1006/bbrc.2000.3104>.
- [56] K. Nishida, Y. Yoshida, M. Itoh, T. Fukada, T. Ohtani, T. Shirogane, T. Atsumi, M. Takahashi-Tezuka, K. Ishihara, M. Hibi, T. Hirano, Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors, *Blood* 93 (1999) 1809–1816, <http://www.ncbi.nlm.nih.gov/pubmed/10068651>.
- [57] T. Brummer, D. Schramek, V.M. Hayes, H.L. Bennett, C.E. Caldon, E.A. Musgrove, R.J. Daly, Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein, *J. Biol. Chem.* 281 (2006) 626–637, <http://dx.doi.org/10.1074/jbc.M509567200>.
- [58] D. Bier, M. Bartel, K. Sies, S. Halbach, Y. Higuchi, Y. Haranosono, T. Brummer, N. Kato, C. Ottmann, Small-molecule stabilization of the 14-3-3/Gab2 protein-protein interaction (PPI) interface, *Chem. Med. Chem.* 11 (2016) 911, <http://dx.doi.org/10.1002/cmdc.201500484>.
- [59] BioSolveIT GmbH, SeeSAR 5.5.